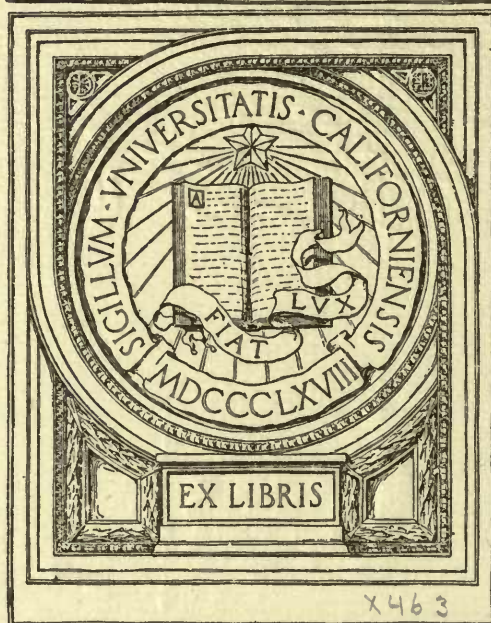


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PARASYNAPTIC STAGES IN THE TESTIS
OF *ANEIDES LUGUBRIS* (HALLOWELL)

BY

HARRY JAMES SNOOK AND J. A. LONG

Master of Science Thesis
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INTRODUCTION

In many of the theories proposed to account for the segregation of hereditary factors a more or less specific association between the factors and the chromatic elements of the cell is assumed. Any judgment as to the tenability of such theories ought to be based, at least in part, upon the behavior of the chromosomes themselves, the phenomena of synapsis being particularly significant in this connection. The present study has been undertaken in an effort to obtain as much evidence as possible about the actual conditions during the synaptic period. Inquiries into the spermatogenesis of amphibians have contributed much towards a solution of the problem, and it has seemed worth while to extend the observations to another representative of the same class. An attempt has been made to answer the following questions: Is there a stage, or a series of stages, in spermatogenesis during which two or more chromosomes unite, or in any manner become very closely associated? If so, in what manner and to what extent does the process take place, and what is the subsequent fate of the elements which have been joined together? This inquiry is limited to the synaptic period and the stages which precede or immediately follow it.

The urodele, *Aneides* (= *Autodax*) *lugubris* (Hallowell), was chosen as the subject of this investigation for the following

reasons: (1) The male sex cells are large; (2) the animal is fairly common in this vicinity and may be obtained throughout the entire year; and (3) the seriation of stages within the testes is easily followed because all phases are frequently found in a single testis, beginning at the anterior end with lobules of spermatogonia and passing posteriorly to a region occupied by mature spermatozoa. Moreover, the cells in any particular lobule appear to have reached approximately the same stage in differentiation.

The material used for this research was obtained within a radius of five miles from Berkeley, with the exception of a few specimens from Twin Peaks, San Francisco, and much of it was secured upon the campus of the University of California. The salamanders may readily be found under stones and logs or in crevices of the rocks. They have also been taken in burrows at least a foot below the surface of the soil, but in all such cases observed the burrow opened beneath a rock. It is difficult to find them during the dry season, as they seek the deeper, moist levels. According to the investigations of Ritter and Miller (1899), they breed in June and July. Although no specimens were collected during June, July, and September, material was obtained during every other month.

A number of fixing fluids and staining processes were tried with varying success. Bouin's, Zenker's, and Flemming's fluids proved most valuable and were extensively employed. Gilson's fluid seemed to damage the cells of the outer cysts; otherwise, some very good late prophase were obtained by its use. The testes were embedded in paraffin and sectioned longitudinally in order to show the seriation to the best advantage. The sections, cut from six to twelve micra in thickness, were mounted on slides in the usual way.

With Bouin's and Zenker's fluids three stains were tried: iron-alum-haematoxylin with orange G and acid fuchsin as counterstains; safranin followed by gentian violet; and phosphotungstic acid haematoxylin. The last seems to be the best for general purposes, as it stains the growing spermatocyte beautifully without overstaining the mitotic figures. It differentiates the structures of the cell very clearly, staining the chromosomes deep blue and the spindles reddish. Many equatorial plates can

be excellently demonstrated with safranin and gentian violet after Zenker's or Bouin's solutions. Material fixed in Flemming's solution was stained either with safranin and gentian violet after the Gram method, or with iron-alum-haematoxylin and orange G. Gilson's fluid was followed by safranin and lightgreen.

A few testes, fixed in Flemming's solution and passed through the alcohols to seventy per cent, were divided into four or five parts which were transferred with a drop of alcohol to slides prepared with egg albumen and then thoroughly crushed and ground up under a cover glass. The seventy per cent alcohol was then drawn off and the albumen coagulated by dropping ninety per cent alcohol upon it, fixing the fragments and loose cells to the glass. The material thus prepared was carried through the alcohols and stained either in iron-alum-haematoxylin without counterstains, or in safranin with gentian violet. While many of the cells were broken up, or flattened out, a considerable number remained intact. Because of their large size they are somewhat opaque, but most of them are clear enough for study. This method destroys every evidence of seriation and for that reason is not of great value by itself, yet, taken in connection with the sectioned material, it has been found very useful. This type of preparation, in which the structures are entire, was most largely used in the determination of the number of chromosomes previous to the first maturation division.

THE SPERMATOGONIA

The anterior portion of the testis is occupied by spermatogonia and occasionally lobules may be seen which are filled entirely, or in part, with cells in the process of division. Among these it is possible to pick out polar views of the equatorial plate. By first carefully drawing them with the aid of a camera lucida, and then counting the chromosomes shown in the resulting sketch, the number and form were determined. It was not always possible to demonstrate each element clearly, owing to the sinuous form of many of the chromosomes and the tendency for them to gather in clumps, more or less masking those below; and in

many cases it could only be discerned that there were more than twenty-five and less than thirty. Discarding the latter as doubtful, there still remained nine clear cases in which the number was definitely twenty-eight, and one case, just as clear, in which it was only twenty-three.

Plate 25, figure 1, represents this latter and plate 25, figure 2, illustrates the more usual condition. They are drawn from cells in one section, located near each other in the same lobule. The two small elements in the center of the plate in both figures are quite characteristic of this stage, and, although they cannot always be found occupying the same relative position, and may sometimes be widely separated, they can usually be identified with chromosomes of the same general size and shape lying within the ring in other spindles. The chromosomes appear to be band-shaped and are usually bent into the form of a letter U with the free ends pointing outward. There is considerable variation in size and shape and in their position with reference to the spindle.

A split can frequently be discerned in the chromosomes of the equatorial plate, as is shown by several elements in the cells figured. It is believed that this is evidence that the plate is fully formed and that division is about to occur. Many dividing cells are situated in the immediate vicinity of these two.

The significance of the lesser number shown in figure 1 is not known, nor is it possible to say just how widely such variations occur. In the case under consideration there is no evidence that the condition is in any sense abnormal, for it does not differ from the others except in the number of chromosomes. This plate lies in a section twelve micra thick, and the following section shows only a few tips in the remainder of the cell.

Although such exceptional cases certainly exist, even a casual exploration of the lobules of dividing spermatogonia will convince one that in a large majority of cases more chromosomes are present than are delineated in figure 1. In view of the fact that in every clear case found, with the exception noted, twenty-eight definite elements could be made out, it seems justifiable to say that the number of chromosomes in the equatorial plate of *Aneides* is usually, but not universally, twenty-eight.

THE SPERMATOCYTE

Following the last spermatogonial telophase, the chromatin becomes very diffuse and is irregularly distributed in the form of clumps or "net-knots" connected by fine threads (pl. 25, fig. 3). If there is any arrangement or order in these threads, it is completely masked by the patches of chromatin, nor is there any suggestion of orderliness in the size or distribution of the latter. There is no clear evidence of a "chromoplast" as described by Eisen (1900) and later by Janssens (1905) in the male sex cells of *Batrachoseps attenuatus*.

Janssens also found evidence of a rotation of the nucleus during this period, whereby the "chromoplast," at first in the neighborhood of the sphere, came to lie opposite it. In *Aneides* the sphere is found at one side of the nucleus, but further than that it has been impossible to demonstrate any relation between it and the elements of the nucleus until the resting stage is past.

In lobules more posterior than those containing the resting nuclei the fine threads gradually become more pronounced and can be followed without difficulty for considerable distances. At the same time it is plain that the threads are of greater diameter and stain more heavily in the region of the sphere than in the rest of the nucleus. The net-knots appear smaller and are comparatively few, especially in the immediate vicinity of the sphere, which may be said to lie at the proximal pole of the nucleus. In this region the threads appear somewhat condensed (pl. 25, fig. 4) and close observation shows that they are associated two by two in such a manner that when viewed from the side they resemble the letter V, with the angle pointing toward the sphere and the diverging arms prolonged in the opposite direction. They are unbranched, as was also found to be true for *Salamandra* by the Schreiners (1906) and for *Batrachoseps* by Janssens (1905), the description of the conditions in *Batrachoseps* being confirmed by Wilson (1912), who examined Janssens' preparations. Sections fixed in Bouin's, Zenker's, or Flemming's solutions and stained in a variety of ways show this peculiarity. At this stage the distal portion of the nucleus shows no such polar orientation, and if the sections should be so cut

as to include this part only it could be distinguished with extreme difficulty, if at all, from the preceding stage.

Comparatively few of the cells examined exhibited the V-figures when observed from the side, the greater number displaying a Y-shaped arrangement of the threads in the vicinity of the proximal pole, with the stem of the Y ending in the immediate neighborhood of the sphere and the arms drawn out into fine threads which are lost in the distal portion of the nucleus (pl. 25, fig. 5). In the part of a section which contains spermatocytes in this condition there is a gradual change from the V- to the Y-figure; the cells characterized by the short-stemmed Y-figures give place gradually to those in which a long-stemmed figure predominates (pl. 25, figs. 6 and 7); and in the individual nuclei there is considerable variation in the length of the structure from the free end to the fork. At times the thick threads seem also to be double, but it is not possible to demonstrate this condition in most cases. As the length of the stems of the Ys increases the extent of the network diminishes.

As can be easily imagined, it is practically impossible to determine with accuracy in this stage the number of the V- and Y-figures, and of the fine threads of which they are composed. While the threads are perfectly clear in side views, they are easily confused when seen from one end. Nevertheless, in one nucleus corresponding to figure 4, so cut that the part containing the pairing threads was viewed from the pole, a diagram indicated the presence of twenty-six to thirty Vs and Ys, or, in other words, of fifty-two to sixty threads; and in another case in a stage like that shown in figure 6 there were clearly twenty-eight thick threads (stems of Ys).

This stage corresponds closely to that figured by Janssens (1905) in his paper on the spermatogenesis of *Batrachoseps* and designated by him the amphitene, in distinction to the preceding or leptotene condition in which the fine threads are not united. Much stress is placed upon it by the Schreiners (1906) in their discussion of the spermatogenesis of *Salamandra maculosa* as the stage in which a conjugation of the chromosomes takes place. Many writers have overlooked it entirely, because, no doubt, of its close resemblance to the leptotene period and the small number

of nuclei usually found in this condition. If the number of amphitene cells can be taken as an indication of the duration of the period, it must be considered as relatively short, because the cells occupy a much smaller proportion of the testis than either the spermatogonia or the cells that have advanced to the next stage.

Posterior to the region in the testis last described are nuclei containing horseshoe-shaped loops, with their free ends turned towards the sphere and their bends in the distal half. They appear somewhat granular (pl. 25, fig. 8 and pl. 26, fig. 10). These loops have been described in a number of urodeles and would appear to be very characteristic of spermatogenesis in salamanders. In thickness they exceed somewhat the thick threads formed by the union of two fine diverging ones. No trace of the latter can now be seen. Cross-sections through the proximal portion of the nucleus show clearly twenty-eight cut ends, demonstrating the number of loops to be fourteen (pl. 26, fig. 11). Polar views show the presence of twenty-eight converging threads with their free ends crowded together in the immediate vicinity of the sphere and very near to the nuclear membrane (pl. 26, fig. 9).

It will be remembered that this polar orientation is also a characteristic of the preceding stage, a lateral view showing the same arrangement of the thick threads forming the stem of the Y-figure as is here presented by the ends of the loops. This resemblance is very marked, indeed, and together with the evidence afforded by seriation leads to the conclusion that the loop is derived from two fine threads which have now completely disappeared as separate threads and have fused into one.

Up to this point the cells have been gradually increasing in volume, a fact which makes seriation more certain; but during the stage of well-developed loops relatively little growth takes place. As the cells in this condition usually occupy a rather large portion of the testis, it seems probable that this period lasts for a much longer time than the amphitene stage.

Later all evidences of polarization are lost and the chromosomes end without apparent relation to the position of the centrosomes or to each other. At the same time a longitudinal split develops, dividing each loop more or less completely into two

portions throughout the greater part of its length, but leaving one or both of the ends intact (pl. 26, fig. 12), a process which is the reverse of the one previously described. Cells in which the split is developing can readily be distinguished from those in the amphitene stage, by the absence of polarization, by their larger size, and by their position in the testes near the region in which the maturation divisions take place. They are separated from the amphitene cells by the extensive and clearly defined region of the polarized loops.

This and the subsequent stages in amphibians have been described so frequently since first pointed out by Fleming (1887) that they need not be discussed in detail here. According to Hermann (1889), Meves (1897), Eisen (1902), Janssens (1901, 1905), Kingsbury (1902), and other careful investigators, the longitudinal halves of the loop are separated in the first maturation division. This seems to be true in the case of *Aneides*. The split loops shorten, thicken, and at the same time become twisted to form what Janssens designates the streptotene stage (pl. 26, fig. 13). They finally form heterotypical chromosomes, the halves of which, corresponding to the halves of the split loops, pass to opposite poles during the following anaphase.

A number of spindles representing the first maturation division were examined and some drawn with the aid of the camera lucida (pl. 26, fig. 14). The number of chromosomes was fourteen, though fifteen were counted in two cases. In the latter, however, from the position of the chromosomes it seems probable that a chromosome had just divided and it is possible that fourteen was the original number in all cases examined. Both sections and entire cells were employed in making these counts, the latter being found particularly valuable. While they are frequently somewhat dense and often pressed out of shape, it is hardly possible that any of the chromosomes could be lost without rupturing the cell membrane. In that case the loss would be detected.

DISCUSSION AND CONCLUSIONS

As previously mentioned, the usual number of chromosomes found in the equatorial plate of the spermatogonial spindle is

twenty-eight. In the stage of the polarized loops, cross-sections and polar views in the neighborhood of the sphere indicate the number of loops to be fourteen, which agrees with the number of chromosomes comprising the equatorial plate of the first maturation division. This difference between twenty-eight and fourteen is to be expected if the chromosomes conjugate whether by the parasynaptic or telosynaptic method.

Meves (1911) avoids the problem of synapsis by stating: "Die Geschlechtszellen bzw. ihre Kerne haben nach meiner Vorstellung (1907) die besondere Eigenschaft ererbt, beim Eintritt in die Wachstumsperiode nur die halbe Zahl von Chromosomen auszubilden." To which Wilson (1912) replies, "Certainly the adoption of this simple solution would save a great deal of trouble; but I fear that the facts compel us to take a more roundabout way out of our difficulties." A condition so well-defined and clear as the amphitene stage would seem to be too significant to be thus lightly considered.

This has been interpreted by different investigators to represent either a union of two threads (the Schreiners (1906), Janssens (1905, 1908), Wilson (1911), or a splitting of one Meves (1908), Goldschmidt (1908), Fick (1908). In *Aneides* the evidence afforded by seriation as recapitulated below seems to the writers to point toward the former interpretation.

1. Fine, unpaired threads become polarized with respect to that side of the nucleus near which lies the sphere, the proximal pole.

2. Coincident with this polarization, threads are found associated two by two at the proximal pole of the nucleus.

3. By the association of any two threads there is formed a figure, which, when observed from a position at right angles to an axis passing through the poles of the cell, resembles the letter V with the angle turned toward the sphere.

4. Seriation shows that the V-shaped figures are succeeded by Y-like figures, of which the stem, frequently exhibiting a double condition, lies in the region of the centrosomes, while the arms diverge widely away from the sphere. Furthermore, it may be observed that as this stage becomes more advanced, the stem of the Y increases in length at the expense of the arms.

5. More posterior in the testes the Y-figures are replaced by loops showing the same polarization, i.e., with the free ends directed toward the sphere while the bends are found in the region opposite to the centrosomes.

6. At this stage the fine threads can no longer be detected and the loops appear as single threads.

This evidence leads to the conclusion that the loops are formed by the union of the fine threads. A more critical examination of the early stages raises the question as to what are the relations of the conjugating leptotene threads to each other. Up to this point the individual V- or Y-figures described in any one nucleus have been considered as unrelated to each other. If there are only fourteen bifid figures in any one nucleus, each must be considered one end of a loop in the later polarized stage, the other end of the loop not yet having come into existence. But since there are about twenty-eight pairs of threads at the beginning of polarization and since the twenty-eight free ends of the completed loops directed toward the proximal pole correspond in position to the stems of the Ys, it leads one to think that the two ends of each loop are formed simultaneously and before the middle part comes into existence.

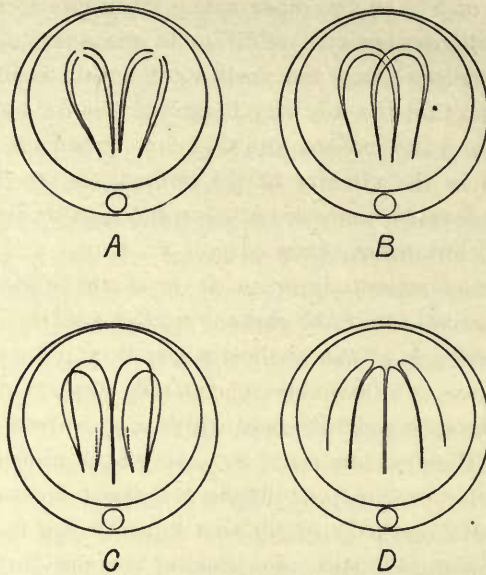
While the foregoing is strong evidence that the stem of each Y-figure becomes one end of a polarized loop, the way in which the two ends of each loop become associated remains to be considered. It will be remembered that each one of the fifty-six short, fine, leptotene threads which unite in one way or another to form the fourteen loops is indirectly continuous with the others through the medium of the network. The further evolution of these threads might be thought of in one of two ways. In the first place the fifty-six threads might be imagined as separate, individual filaments which pair to form the twenty-eight Y-figures; in the second place, they may be conceived of as not so many independent parts, but as so definitely related that each branch of each Y would be one end of a potential thread, the other end of which would be represented by one of the branches of some other Y.

In regard to the first of these possibilities, it might be argued that the arms of the Y-figures on one side meet and join with

similar arms of Y's on the other side, giving rise to the loop by a process of telosynapsis in addition to parasynapsis (fig. 4). That there is no evidence for such a conclusion is indicated by the observation that threads may frequently be followed for long distances around the nucleus and that free ends have never been found except in the vicinity of the sphere. When the threads are definitely formed, there are no morphological indications that an end-to-end union has taken place.

These considerations indicate at once the validity of the second alternative, for if the threads exhibit no free ends except at the proximal pole of the nucleus where they unite to form the Y-figures, it seems reasonable to conclude that they represent the ends of twenty-eight threads which are evolved from their ends towards their middle at the expense of the nuclear network. In other words, each of the fifty-six threads becomes directly continuous with one only of its own kind by the time that the loops are complete. If this conception of their origin and nature be sound, the way in which they become associated in the loops might be interpreted in one of two ways: (1) The loops may be formed by the association of two potential threads by a process which begins at approximately the same time at both ends (fig. B). (2) One leptotene thread might be joined at the ends to two other threads (fig. C). Both of these interpretations have one feature in common, that of a side-to-side union. There seems, however, to be little reason to believe that the second condition actually exists in the male sex cells of *Aneides*. If each leptotene thread were joined at the ends to other threads at the beginning of polarization (fig. C), as the bifid figures closed up, most of the threads would be drawn together in one or more points in the region of the distal pole of the nucleus (fig. D), which is, in fact, the region of widest separation. The fact that there is no evidence of such behavior upon the part of the threads would seem to dispose of this interpretation.

Therefore, it seems probable that the number of pairing threads is twenty-eight and that, as rapidly as they are evolved, they unite two by two, side by side, and at both ends, the middle part of the potential threads being lost in the distal half of the nucleus.



Figs. *A* to *D*.—Diagrams to illustrate possible modes of association in synapsis of the fifty-six fine threads (leptotene) which are formed from the nuclear network and become polarized with regard to the sphere which lies at the proximal side of the nucleus. The large outer circle represents the cell wall; the large inner, the nuclear membrane; the small one between the two large, the sphere.

Fig. *A*.—Of the fifty-six threads which are imagined as separate, distinct filaments eight are represented as forming two loops (amphitene) by a double process of pairing. They form by parallel union four (of the twenty-eight) Ys which by end to end junction give rise to the loops. There is no evidence of such a condition.

Fig. *B*.—Four complete threads are indicated, the eight ends of which, corresponding to the eight separate threads of figure *A*, arise separately, are at first merged into the nuclear network of the distal part of the nucleus, and with the disappearance of the network become continuous as four threads. These before completion and while potentially present in the network become paired in such a manner that the ends of two threads unite to form two Ys, which by closing up give rise to a single loop which is unconnected with the other. Fourteen such loops are formed.

Fig. *C*.—The complete threads arise as in figure *B*. A different mode of synapsis is represented in which the two ends of one complete thread are not paired with the two ends of only *one* other thread, but with one end each of *two* other threads.

Fig. *D*.—A later stage of the condition shown in figure *C*, showing how with the final closing of the Ys the bends of the loops would be drawn together, a condition not existing.

With these facts and considerations in mind, it is concluded that the V- and Y-figures do not indicate a splitting, as maintained by some, but that they represent a progressive, parallel union or conjugation of fine (leptotene) threads; in other words, a parasynaptic union. This union takes place during the amphitene stage and leads to the formation of the single, thick, polarized loops. There is no reason for confusing the split which does occur in the stage following the formation of the loops (and which may be the reverse of parasynapsis) with the union in the amphitene stage, since the two stages are easily distinguishable both by their appearance and position in the testis.

The evidence set forth above does not warrant an assertion that the conjugating leptotene threads are identical with the spermatogonial chromosomes. Nevertheless, the fact that the number of chromosomes (tetrads) in the first maturation spindle is half that of the chromosomes in the spermatogonial division, together with the evidence that the tetrads are formed by the union of threads evolved from the nuclear network which in turn is formed from the chromosomes of the last spermatogonial telophase, suggests very strongly that the spermatogonial chromosomes which went into the nuclear network reappear as the pairing leptotene threads. This idea is further supported by the manner in which the two branches of two Ys become continuous as though they were the ends of threads (chromosomes) potentially existing in the network, though not distinguishable as such. Or, to reverse the conception, if it is considered that the spermatogonial chromosomes retain their continuity throughout the resting stage of the nucleus, then it is easier to comprehend why the proper Y-figures become associated as the leptotene threads are evolved, for the chromosomes, though in modified form and only partly distinguishable as threads, begin to pair as they might if pairing were delayed until they were completely formed. Although this way of stating the conception is open to the criticism that it is an argument in a circle, still the conception is worthy of consideration as having some weight on the positive side of the question of the individuality of the chromosomes.

It has already been pointed out that the stage of the polarized loops which follows that of the conjugation of the leptotene

threads has a relatively much longer duration than any other of the stages of the prophase of the first maturation division. If it is true that the conjugating threads have the value of paired maternal and paternal chromosomes and constitute the mechanism for the segregation and recombination of mendelian characters or their factors, it is to be noted that there is ample opportunity for the reconstitution of the chromosomes and for any interchange of substances composing the chromosomes which may be concerned in the transmission of inherited characters.

SUMMARY

1. The usual number of chromosomes found in the spermatogonia of *Aneides lugubris* is twenty-eight.
2. The V-figures at the beginning of polarization number twenty-six to thirty, and Ys at a slightly later stage twenty-eight.
3. The number of polarized loops and of tetrads formed from the loops is fourteen.
4. Each tetrad is the result of a parasynaptic union of fine threads.

Transmitted May 3, 1913.

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PLATE 25

All the figures are drawn at the same magnification (x 2350) with a Spencer 2 mm. objective, a Zeiss No. 12 compensating ocular, and an Abbe camera lucida. Reduced magnification on plates, 1700 diameters.

Fig. 1. Spermatogonium in the equatorial plate stage, showing twenty-three chromosomes. Fixed in Zenker's fluid and stained with iron-alum haematoxylin and orange G.

Fig. 2. Same stage as above but with the usual number of chromosomes (twenty-eight). Zenker's fluid, iron-alum haematoxylin, and orange G.

Fig. 3. Spermatocyte before the beginning of polarization. Fixed in Zenker's solution and stained with phosphotungstic acid haematoxylin.

Fig. 4. Spermatocyte at the beginning of polarization showing V-figures. Zenker's fluid and phosphotungstic acid haematoxylin.

Fig. 5. Spermatocyte with short-stemmed Y-figures. Phosphotungstic acid after Zenker's fluid.

Figs. 6 and 7 illustrate successive steps in the pairing of the threads. The section from which Fig. 6 was taken was fixed in Zenker's solution and stained with phosphotungstic acid haematoxylin. Figure 7 was taken from material fixed in Bouin's fluid and stained with iron-alum haematoxylin.

Fig. 8. A lateral view. Loops completed. Zenker's fluid and phosphotungstic acid haematoxylin.

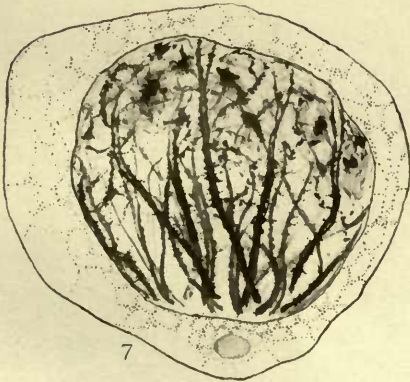
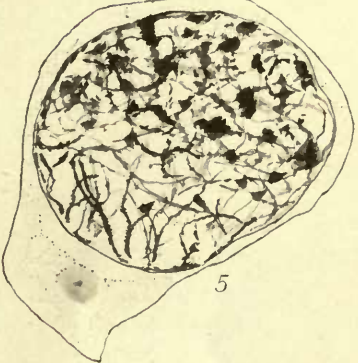
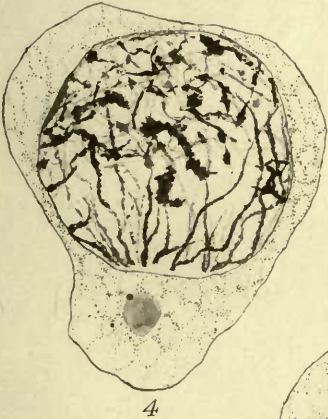
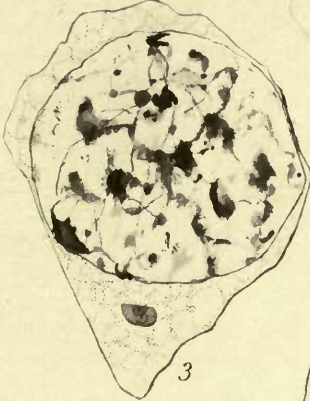
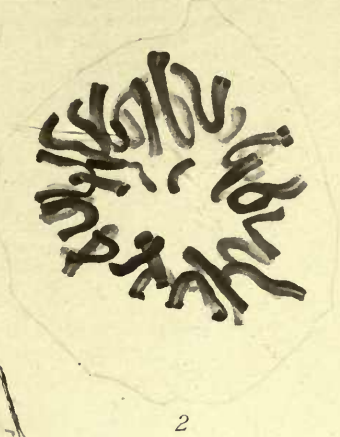
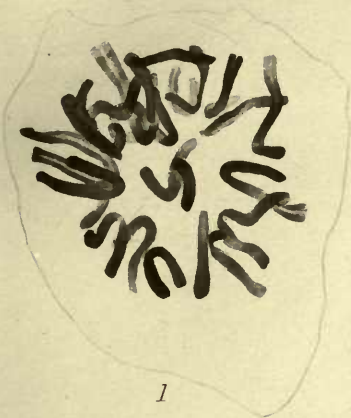


PLATE 26

Fig. 9, 10, and 11. The loops completed.

Fig. 9. Pole view. The sphere lies above the nucleus. The twenty-eight free ends in the vicinity of the sphere represent fourteen loops. Zenker's fluid and phosphotungstic acid haematoxylin.

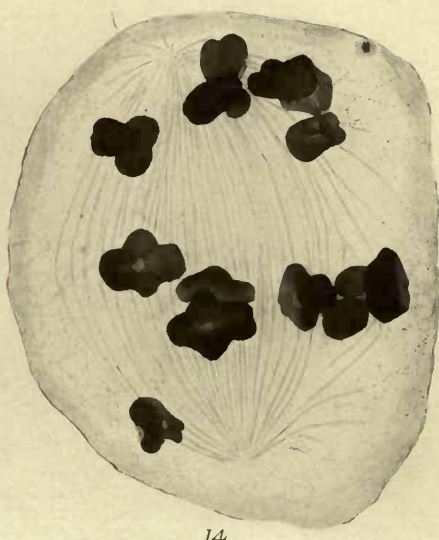
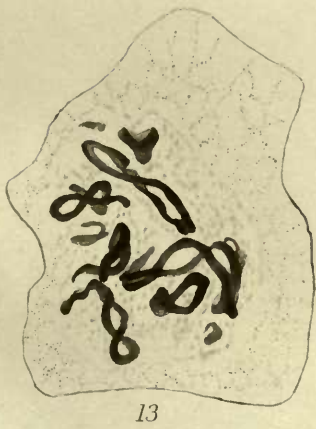
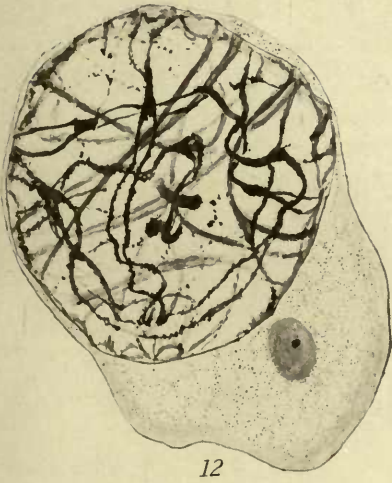
Fig. 10. Lateral view. Zenker's fluid, iron-alum haematoxylin, and orange G.

Fig. 11. Section of spermatocyte in the loop stage. The loops are cut twice. Fixed in Zenker's fluid and stained with iron-alum haematoxylin.

Fig. 12. Complete cell from crushed preparation showing the beginning of the split. Flemming's fluid and iron-alum haematoxylin.

Fig. 13. "Strepsinema." Only a small portion of the cell included in the section. Gilson's fluid, iron-alum haematoxylin, and orange G.

Fig. 14. Prophase to first maturation division. Complete cell from crushed preparation—somewhat flattened. Flemming's fluid and iron-alum haematoxylin.



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